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Biomarker Discovery and Mechanistic Studies of Prostate Cancer Using Targeted Proteomic Approaches

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14. ABSTRACT
Our findings reveal that EMMPRIN immunoreactivity was primarily detected among the glandular epithelial cells; EMMPRIN levels progressively increased with increasing age of TRAMP mice (6-27wks); with the highest detected in liver metastases. Quantitative analysis revealed that by 27-wks (an age exhibiting highly aggressive prostate tumor phenotype), EMMPRIN expression increased significantly (P=0.001). These results suggest that EMMPRIN may have diagnostic value in prostate cancer detection in advanced disease.

15. SUBJECT TERMS
EMMPRIN, prostate cancer, metastasis

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Introduction: The focus of this collaborative work has been the identification of EMMPRIN, a membrane protein found to be overexpressed in prostate cancer epithelial cells that exhibit a highly metastatic potential. Previous evidence identified the involvement of EMMPRIN in cancer development and progression via controlling extracellular matrix remodeling and anchor independent growth by stimulating MMP production, angiogenesis via VEGF by activation of AKT-PIK3 pathway, and cell invasion by up-regulation of urokinase-type palsminogen activator. Thus the emerging rationale on pursuing the role of EMMPRIN as a functional biomarker in prostate cancer metastasis.

Body: Targeting of tumor cell metastasis is of major therapeutic significance and its exploitation may lead to the identification of effective new modulations such as : (**1**) reversing the ability of tumor cells of becoming resistant to anoikis, therefore making them more susceptible to anoikis-inducing agents; (**2**) interfering with the seeding process of tumor cells into secondary places by making tumor cells non-sensitive to the chemotatic and environmental cues of the new target organ; and (**3**) making these secondary targets less "appealing" to the cancer cells by blocking key molecules promoting cancer cell seeding and survival. Membrane proteins play a critical role during the metastasis process since they regulate cell-cell interactions and coordinate cell-tumorenvironment communication. The initiating PI supported by this PCRP Synergistic Grant (USAMRMC PC074317), Dr. Zhu by utilizing proteomic approaches, identified EMMPRIN as one of differentially expressed membrane proteins in prostate cells, revealing considerably higher levels of EMMPRIN protein in highly metastatic human prostate cancer cells.

   (1) Ongoing Work: Ongoing studies focus on a) the mechanistic dissection of EMMPRIN's contribution to metastasis and b) the significance of EMMPRIN in human prostate cancer progression to metastastic disease and clinical

outcomes to define the potential value of this player as a marker of metastasis, studies will pursue expression profiling of EMMPRIN proteein levels in a series of human prostate cancer specimens of increasing Gleason grade and metastastic lesions. Human prostate tissue specimens from patients with primary and metastatic prostate tumors (Department of Pathology, University of Pittsburgh), will be subjected to immuno-profiling for EMMPRIN expression and quantitative analysis will be achieved using computer-image analysis in normal prostate; benign prostate hyperplasia, BPH; prostate primary tumors (Gleason Score range 6-9); and metastatic lesions (n=45). Additional translational studies will focus on establishing a correlation between EMMPRIN expression with serum PSA levels, Gleason grade and patient (disease-free) survival in a large cohort of prostate cancer patients, which may define the value of EMMPRIN as a cancer metastasis marker.

(2) Ongoing experiments investigate the expression of a critical tight junction protein, ZO-1 in prostate tumors with increasing grade. Preliminary staining revealed clear striations of Tight Junctions visualized in epithelial regions that are strongly detected in low-grade tumors and expression is decreased with increasing age of the TRAMP mice. Prostate tumors from 20, 24, 27 and 31-week-old mice are currently being interrogated for tight junction protein expression that will be correlated with the EMMPRIN expression (an inverse correlation is expected).

(3) Experiments will be pursuing the consequences of EMMPRIN loss/silencing in prostate cancer cells on the transcriptional regulation of the major players of the process of Epithelial Mesenchymal Translation (EMT). The prostate tumor microenvironment represents a key component of the invasive dynamic of prostate cancer. In reference to this new exciting direction of the work supported by this program, please see as Appendix 1, a paper published by Dr. Kyprianou's group, an original article [(Zhu and Kyprianou, The FASEB Journal, 24: 769-777, 2010)] demonstrating the ability of androgens to induce EMT of prostate cancer epithelial cells.

Key Research Accomplishments: In our collaborative interaction we showed that EMMPRIN loss in human prostate cancer cells, had no significant consequences on prostate cell growth, proliferation or apoptosis. We found, however, a significant suppression in prostate tumor cell invasion, migration and metastatic ability using in vitro assays. These data are reported in the manuscript to be published in the Prostate (please see as Appendix 2, a copy of proof-print of the manuscript by Zhu et al, 2011).

My contributions at the translational level have been the determination of the potential predictive value of EMMPRIN in prostate cancer progression, first utilizing the TRAMP mouse model of prostate tumorigenesis and subsequently analyzing a series of human prostate cancer specimens of increasing Gleason grade. The TRAMP mice (C57BL/6J) are transgenic mice that express SV40T/t antigens under the prostate specific rat probasin promoter. TRAMP transgenic males develop prostate adenocarcinoma in a manner resembling the clinical progression of human prostate cancer from intra-epithelial neoplasia to androgen-independent metastatic tumors. Hematoxylin and eosin (H&E)-stained sections of prostate tissues from TRAMP/+/+ male mice were evaluated (by N.K.) to confirm pathological grade. Prostate sections from wild type and the TRAMP tumors of increasing grade and metastatic lesions (5μm), were subjected to immunohistochemical analysis for EMMPRIN expression. Slides are examined under a fluorescence microscope and expression is determined in a semiquantitative fashion, incorporating both the staining intensity and the number of positively stained epithelial cells. As shown in Figure 1 histopathological grading of prostatic tumors revealed that in the majority of 16wk-24wk-old TRAMP mice, prostate adenocarcinoma was evident (16-20weeks),and with increasing age (24 weeks), poorly differentiated tumor foci were detected with focal cribriform lesions protruding into the lumen (grade 3-5), representing tumor progression to advanced disease. A score for each histological grade (H) was determined as the product of intensity and proportion (H = I × P).

Reportable Outcomes: The expression profile of EMMPRIN expression was assessed during *in vivo* prostate tumorigenesis in the TRAMP model of prostate cancer progression is shown on Figure 2. Comparative profiling of EMMPRIN expression in normal prostate glands from wild type is comparatively shown. Our findings reveal that EMMPRIN expression is very low or not all present in the prostates from wild type mice, while strong EMMPRIN immunoreactivity is primarily detected among the glandular

epithelial cells of prostate tumors from TRAMP mice (Figure 2). Significantly enough EMMPRIN levels progressively increased with increasing age of TRAMP mice (6-27wks). Quantitative analysis of the immunoreactivity profile for EMMPRIN revealed that by 27-wks (an age exhibiting highly aggressive prostate tumor phenotype), EMMPRIN expression increased significantly (P=0.001). These results suggest that EMMPRIN may have diagnostic value in prostate cancer detection in advanced disease.

Preliminary analysis of the expression of EMMPRIN in human specimens revealed the low immunoreactivity in premalignant lesions of the prostate, i.e. high grade Prostatic Intraepithelial Neoplasia (Figure 3, panel A). Significantly enough, the significance of the tumor microenvironment, as dictated by EMMPRIN in promoting the metastatic spread, is highlighted in the immunostaining profile among the epithelial benign areas of a human prostate tissue, adjacent to tumor foci that are totally negative for EMMPRIN (Figure 3, Panel B).

Conclusion: The work on EMMPRIN in prostate cancer is of translational significance as targeting of tumor cell metastasis holds considerable promise in cancer therapeutics and detection. Functional exploitation of EMMPRIN in its ability to dictate prostate cancer cell metastatic behavior in the context of the tumor microenvironment, may lead to new approaches for impairing the metastatic process by : a) reversing the ability of tumor cells to resist anoikis (thus enhancing their sensitivity to anoikis-inducing agents); and b) interfering with the tumor cell migration and adhesion to secondary sites. Our findings demonstrate that EMMPRIN loss has a major impact on cell membrane re-organization and spatial disruptions that significantly affect prostate tumor cell adhesion, migration and invasion. As the project matures, it enables new insights into the function of EMMPRIN as a contributor to prostate cancer cell metastastic behavior and its potential as a therapeutic target during tumor progression to metastasis in vitro and in an *in vivo* model of prostate tumorigenesis. Ongoing studies focus on immunoprofiling EMMPRIN expression in human prostate specimens from patients with

primary and metastatic tumors to determine the significance of EMMPRIN as a marker

of progression to advanced castration-resistant disease.

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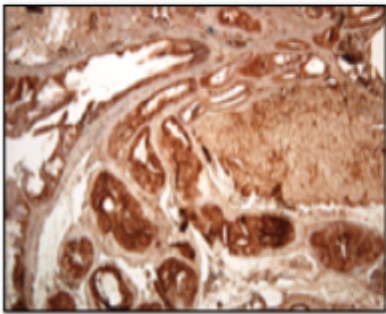
Figure 1

Immunohistochemistry Stain: EMMPRIN distribution in TRAMP+ Mouse Prostate
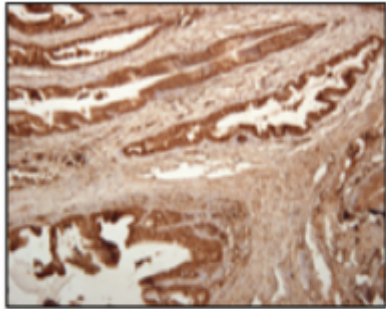
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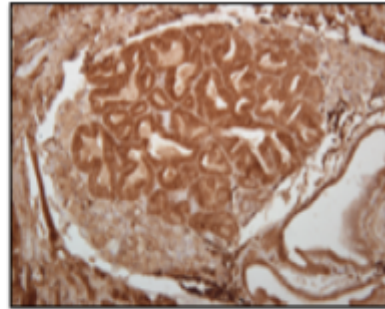
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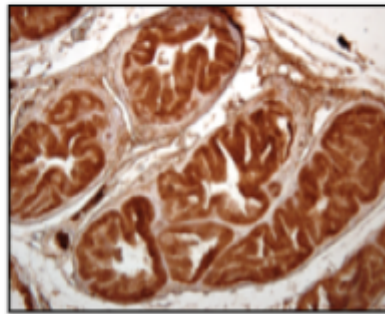
24 Week

Figure 2. Comparative analysis of EMMPRIN expression in normal prostate and TRAMP model of prostate tumorigenesis. Strong immunoreactivitiy is detected at 27-wks TRAMP mice.

Figure 3

Panel A: Weak EMMPRIN immunoreactivity is detected in areas of High-Grade Prostatic Intraepithelial Neoplasia (HGPIN) in human prostate cancer.

Panel B: Significance of prostate tumor microenvironment. Strong EMMPRIN expression among prostate benign epithelial cells adjacent to tumor glands (negative).

<u>Appendix 1</u>

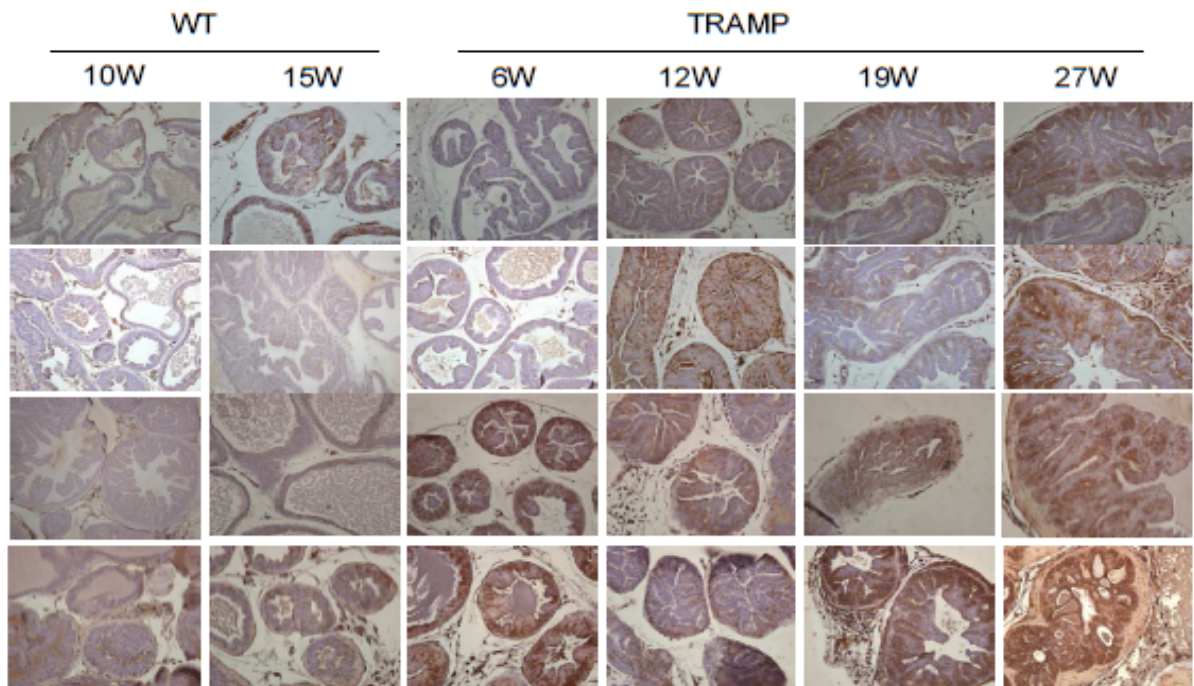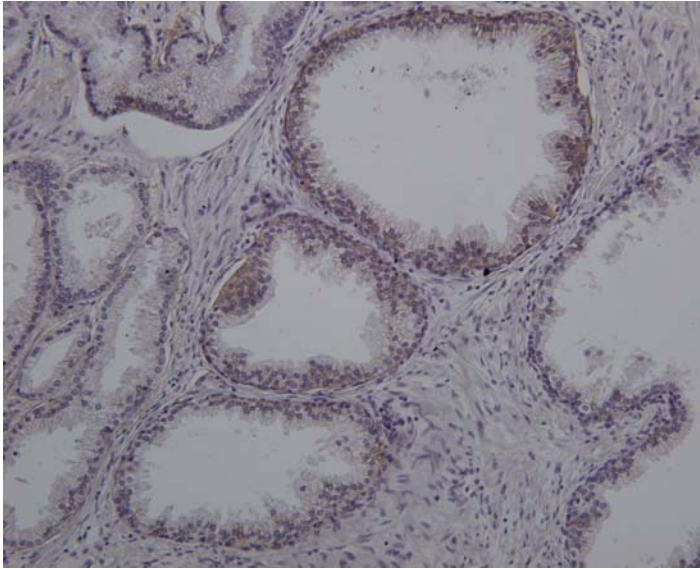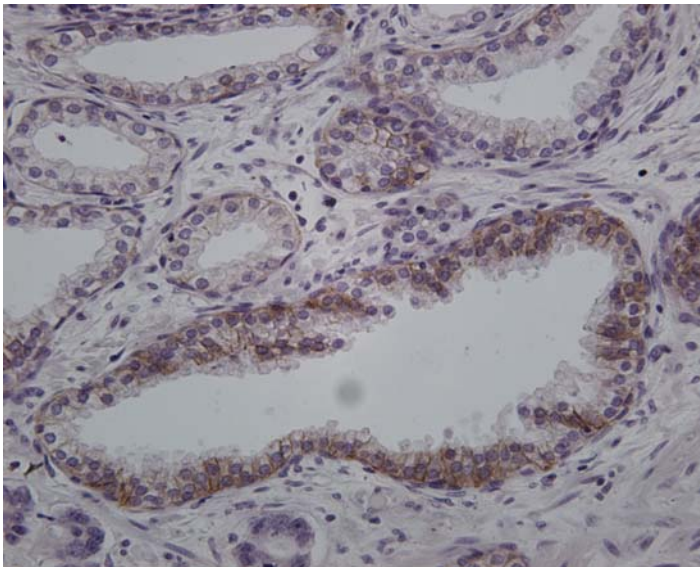# Role of androgens and the androgen receptor in epithelial-mesenchymal transition and invasion of prostate cancer cells

Meng-Lei Zhu*,[†] and Natasha Kyprianou*,[†,‡,1]

*Department of Toxicology, [†]Department of Surgery/Urology, and [‡]Department of Molecular Biochemistry, University of Kentucky College of Medicine, Lexington, Kentucky, USA

ABSTRACT   Androgens are functionally required for the normal growth of the prostate gland and in prostate tumor development and progression. Epithelial-mesenchymal-transition (EMT) is an important process during normal development and in cancer cell metastasis induced by factors within the microenvironment, such as transforming growth factor-β (TGF-β). This study examined the ability of androgens to influence EMT of prostate cancer epithelial cells. The EMT pattern was evaluated on the basis of expression of the epithelial markers E-cadherin/β-catenin, and the mesenchymal markers N-cadherin, as well as cytoskeleton reorganization in response to 5α-dihydrotestosterone (DHT; 1 nM) and/or TGF-β (5 ng/ml). Overexpressing and silencing approaches to regulate androgen receptor (AR) expression were conducted to determine the involvement of AR in EMT in the presence or absence of an AR antagonist. Our results demonstrate that androgens induce the EMT pattern in prostate tumor epithelial cell with Snail activation and lead to significant changes in prostate cancer cell migration and invasion potential. Expression levels of AR inversely correlated with androgen-mediated EMT in prostate tumor epithelial cells, pointing to a low AR content required for the EMT phenotype. These findings indicate the ability of androgens to induce EMT by potentially bypassing the functional involvement of TGF-β, thus contributing to metastatic behavior of prostate cancer cells.—Zhum, M.-L., Kyprianou, N. Role of androgens and the androgen receptor in epithelial-mesenchymal transition and invasion of prostate cancer cells. *FASEB J.* 24, 769–777 (2010). www.fasebj.org

PROSTATE CANCER IS THE MOST frequently diagnosed nonskin cancer and the third leading cause of cancer mortality among men in the United States (1). Prostate cancer mortality is primarily due to failure to cure patients with metastatic disease. In the initial stages, prostate cancer is dependent on androgens for growth and can be suppressed by androgen deprivation therapy (ADT) (2). Prostate tumors, however, eventually recur due to a transition from androgen-dependent to an androgen-independent state leading to highly metastatic disease for which there is no effective therapy available. Androgen action proceeds *via* an axis involving testicular synthesis of testosterone, its transport to target tissues, and its conversion by 5α-reductase to the active metabolite 5α-dihydrotestosterone (DHT). Androgens exert their biological effects by binding to the androgen receptor (AR) and inducing its transcriptional activity. The 5α-reductase enzyme is present in the urogenital sinus before and during prostate development (3, 4), and its inhibition during fetal development results in partial prostate development (5). In adult males, androgens promote secretory epithelial cell survival, the cells primarily transformed in tumor development (6). Androgen deprivation is the only clinically effective therapy for advanced prostate cancer; however, because of the relapse of castration-resistant androgen-independent tumors, the long-term benefit of androgen deprivation in patients with metastatic disease has been debated (7–9).

The process of epithelial-mesenchymal transition (EMT) is a critical event during embryonic development, required for morphogenetic movements during parietal endoderm formation, gastrulation, and formation of organs and tissues (*e.g.*, neural crest, heart, and craniofacial structures) (10). A growing body of recent evidence links EMT to tumor progression and metastasis. Loss of epithelial-cell markers (*e.g.*, E-cadherin and β-catenin) and gain of mesenchymal-cell markers (*e.g.*, N-cadherin and vimentin), particularly at the leading edge or invasive front of solid tumors, has been reported in human tumor specimens and is associated with tumor progression to metastasis (11). Epithelial tumor cells lose cell polarity and cell-junction proteins and at the same time acquire protein mesenchymal-cell markers (*e.g.*, N-cadherin and vimentin) and signaling activities associated with mesenchymal cells facilitating migration and survival in an anchorage-independent environment and ultimately metastasis (11, 12). Pathological EMT in tumor cells results from transcriptional reprogramming of abnormal survival signals *via* receptors, such as platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR),

transform growth factor-β receptor (TGF-βR), and insulin-like growth factor-1 receptor (IGF-1R); and regulatory kinases, such as PI3K, AKT, and mTOR (13, 14). TGF-β is a potent EMT inducer in normal development and organ homeostasis, as well as during tumor progression (15). TGF-β induces EMT *via* Smad-dependent and Smad-independent transcriptional pathways (16). Thus Smad-mediated induction of *Snail, Slug*, and *Twist via* HMGA2 (high-motility group A2) and Smad-independent phosphorylation of Par6 contribute to dissolution of cell-junction complexes (17, 18). Furthermore, EMT recruits the cooperation between oncogenic Ras and receptor tyrosine kinases (RTKs) to induce downstream Raf/MAPK signaling associated with tumor progression and poor clinical diagnosis (19).

We previously demonstrated a functional interplay between androgens and TGF-β signaling toward enhanced apoptosis in androgen-sensitive prostate cancer cells (20). Because tumor epithelial cells gain the ability to migrate and invade by dedifferentiating through activation of biological pathways associated with EMT, the present study investigated the involvement of the androgen signaling axis in EMT and invasive phenotype of prostate cancer cells. We report that androgens induce changes characteristic of EMT and cytoskeleton reorganization, involved in the metastatic behavior of castration-resistant prostate cancer cells.

MATERIALS AND METHODS

Cell lines and transfections

The androgen-sensitive and TGF-β-responsive human prostate cancer LNCaP TβRII cells (generated in our laboratory) (21, 22) and the parental LNCaP, CW22, and PC-3 prostate cancer cell lines were used. The human breast cancer MCF-7 cells and the human renal cancer 786-0 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). To determine the effects of DHT (Sigma-Aldrich, St. Louis, MO, USA) and TGF-β (R&D Systems, Minneapolis, MN, USA), cells were grown in DMEM or RPMI 1640 with 10% FBS (without phenol red) and transferred to medium 5% charcoal strip serum (CSS) before treatment. Casodex was a generous gift from Dr. Chendil Damodaran (University of Kentucky College of Health Sciences). Subconfluent cultures of PC-3 or LNCaP cells were transfected with the pCDNA-Zeo AR vector or AR shRNA vector (Open Biosystems, Huntsville, AL, USA), using the Lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA, USA). pCDNA-zeo AR construct was prepared by cloning the full AR fragment from pCMV5-AR vector (*Bam*HI/*Xho*I). pCMV5-AR was a generous gift from Dr. Donald Tindall (Mayo Clinic, Rochester, MN, USA). After transfection (exposure to plasmid DNA for 6 h at 37°C, 5% CO$_2$), the growth medium was changed to 10% FCS for 48 h, prior to selection in antibiotic-containing medium (25 μg/ml zeosin/puromycin; Invitrogen, Grand Island, NY, USA). Individual colonies were selected, cloned, and grown in 10% FCS-containing medium. Protein expression of transfected AR was examined by Western blot analysis.

Wounding assay

Cell cultures (80% confluency) were subjected to wounding as described previously (23). The number of cells migrating to the wounding area was counted at the end points indicated.

Flow cytometry analysis

Prostate cancer cells (1×10^6) were labeled using a specific primary antibody and stained by fluorescent-conjugated secondary antibody. Samples were subjected to fluorescence analysis using the Partec system (Partec GmbH, Münster, Germany).

Western blot analyses

Total cellular protein was extracted from the cell pellets by homogenization in RIPA buffer. Protein samples (20–60 μg) were loaded on 4%/12% SDS-PAGE gels and subjected to electrophoretic analysis and subsequent blocking. Membranes were incubated with the primary antibody (overnight at 4°C) and the relevant secondary antibodies (1 h at room temperature). The E-cadherin, β-catenin, vimentin, and Parp antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA); The AR, tubulin, and N-cadherin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); The cofilin and α-actin antibodies were purchased from Sigma-Aldrich. The antibody against talin1 was a generous gift from Dr. R. McCann (Mercer College, Macon, GA, USA); The GAPDH antibody was purchased from Novus Biologicals (Littleton, CO, USA).

Invasion assay

The invasion ability of prostate cancer cells was determined using the transwell chamber assay. Matrigel (1 mg/ml) in serum-free cold cell culture medium was placed in the upper chamber of a 24-well transwell and incubated for 5 h at 37°C. Cells were harvested, and cell suspensions (100 μl) were placed on the matrigel, and the lower chamber of the transwell was filled with culture medium in the presence of 5 μg/ml fibronectin, as an adhesive substrate. DHT (1 nM) was added in both upper and lower chambers. Following 48 h of incubation at 37°C, transwells were removed and stained with Giemsa solution. Noninvading cells on top of transwells were removed, and invading cells were counted under the microscope.

Immunofluorescence staining

Cells were plated (1×10^5 cells/well) in chamber slides, and after 24 h, cells were incubated with RPMI 1640 + 10% CSS supplemented with either DHT (1 nM), TGF-β (5 ng/ml), or the combination of DHT (1 nM)-TGF-β (5 ng/ml), as indicated. Following treatment, cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.1% Triton X-100 in PBS. Cells were stained by incubation with the primary antibody (overnight at 4°C), followed by exposure to the secondary immunofluorescence antibody and FITC-phalloidin, (1 h at room temperature). FITC-phalloidin was purchased from Sigma-Aldrich . Slides were mounted by Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

RNA extraction and real-time RT-PCR

RNA samples extracted with TRIzol reagent were treated with RNase-free DNase I and reverse transcript to cDNA (Bio-Rad, Hercules, CA, USA). Taqman real-time RT-PCR analysis of the cDNA samples was conducted in an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the specific primers of E-cadherin and Snail (Applied Biosystems).

Statistical analysis

One-way analysis of variance (ANOVA) was performed using the StatView statistical program (SAS Institute, Cary, NC, USA) to determine the statistical significance between values. All numerical data are presented as mean ± SE values. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of androgens on EMT pattern of prostate cancer cells

Exposure of PC-3 prostate cancer cells to DHT results in reduced expression of the epithelial markers, E-cadherin and β-catenin, and induction of the mesenchymal marker, N-cadherin expression (**Fig. 1A**), changes characteristic of EMT. The androgen-sensitive LNCaP cells did not exhibit the same sensitivity as PC-3 cells to DHT-induced EMT; a significant reduction in E-cadherin and β-catenin was detected only after exposure to high doses of DHT (10 nM) (Fig. 1A). However, the presence of TGF-β receptor II (TGF-βRII) sensitizes LNCaP prostate cancer cells to the androgenic effect on EMT (0.1 nM DHT) (Fig. 1A). Since nuclear translocation of β-catenin has been established as a significant event in EMT (24, 25), we subsequently analyzed the cytosolic and nuclear fractions of three different cell lines, LNCaP, LNCaP

TβRII, and C4–2B; we found that DHT triggered a marked nuclear translocation of β-catenin only in LNCaP TβRII cells. Consistent with the E-cadherin expression pattern (Fig. 1A), DHT (1 nM) failed to trigger β-catenin nuclear translocation in either the LNCaP or the C4–2 cells (Fig. 1B). To determine the transcriptional modulation of E-cadherin by DHT, quantitative PCR analysis was performed, and down-regulation of E-cadherin mRNA levels was detected in both PC-3 and LNCaP TβRII cells (Fig. 1E).

In view of the widely acknowledged role of TGF-β as a potent EMT inducer, the effect of TGF-β on prostate cancer cell EMT was examined as a positive/reference control. Exposure to DHT alone or in combination with TGF-β led to comparable reduction in E-cadherin and β-catenin levels in LNCaP TβRII cells (Fig. 1C). Because EMT is driven by the transcriptional factor *Snail*, which is up-regulated by TGF-β (16, 26), we subsequently investigated the effect of DHT on *Snail* expression. As shown in Fig. 1D, treatment of LNCaP TβRII cells with DHT alone or in combination with TGF-β led to a significant increase in *Snail* expression. Furthermore, a marked induction in *Snail* expression by DHT was detected at the mRNA level in both LNCaP TβRII and PC-3 cells (Fig. 1F).

Androgens affect cytoskeleton reorganization in prostate cancer cells

The process of cytoskeleton reorganization, which directly affects cell migration and metastatic ability, is a character-
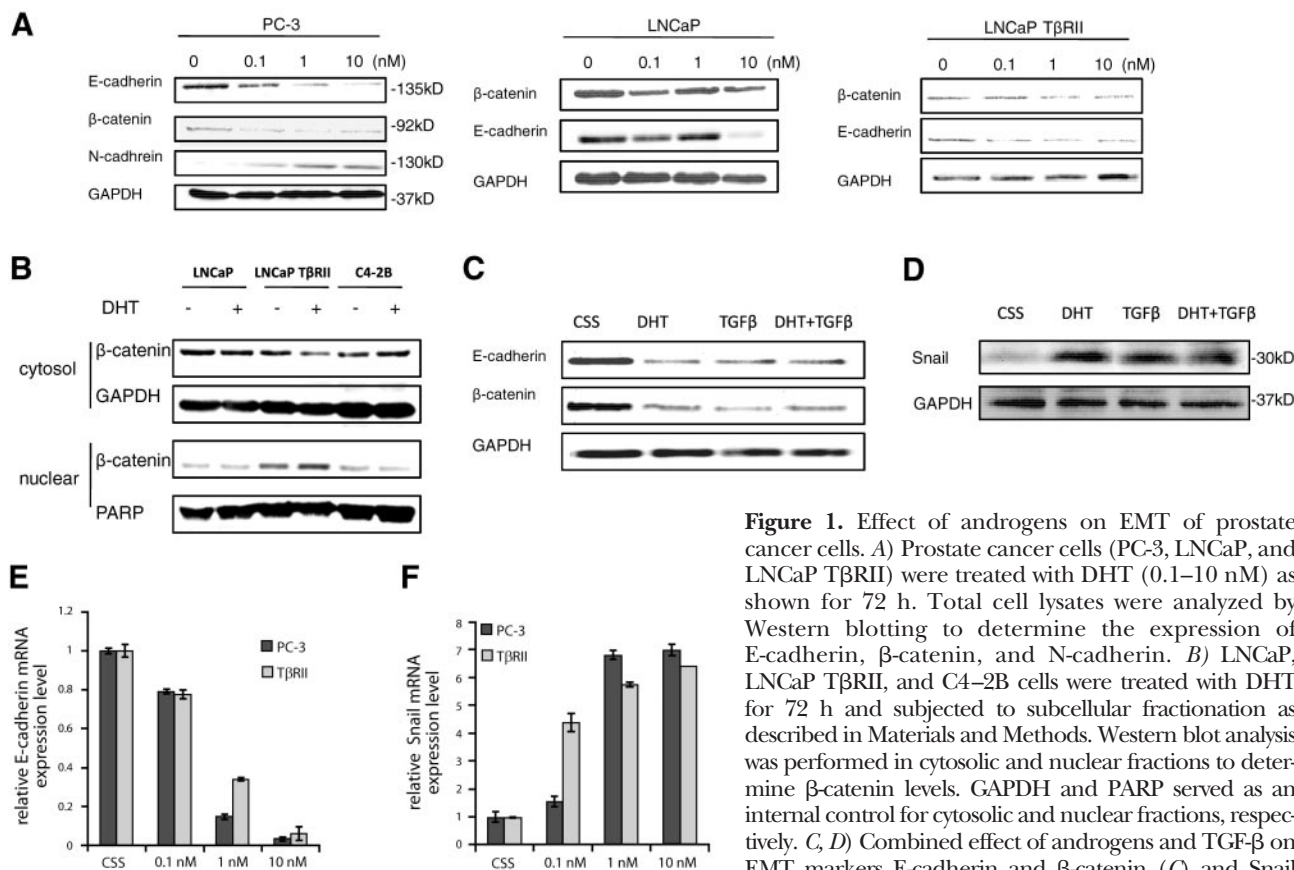

Figure 1. Effect of androgens on EMT of prostate cancer cells. *A*) Prostate cancer cells (PC-3, LNCaP, and LNCaP TβRII) were treated with DHT (0.1–10 nM) as shown for 72 h. Total cell lysates were analyzed by Western blotting to determine the expression of E-cadherin, β-catenin, and N-cadherin. *B*) LNCaP, LNCaP TβRII, and C4–2B cells were treated with DHT for 72 h and subjected to subcellular fractionation as described in Materials and Methods. Western blot analysis was performed in cytosolic and nuclear fractions to determine β-catenin levels. GAPDH and PARP served as an internal control for cytosolic and nuclear fractions, respectively. *C, D*) Combined effect of androgens and TGF-β on EMT markers E-cadherin and β-catenin (*C*) and Snail protein expression (*D*). *E, F*) Results from the real-time PCR analysis of E-cadherin and Snail mRNA levels. LNCaP TβRII and PC-3 cells were treated with DHT (0.1–10 nM) for 24 h, and relative mRNA expression levels of E-cadherin (*E*) and Snail (*F*) were evaluated as described in Materials and Methods.

istic phenomenon in EMT. One of the critical proteins that promotes actin polymerization and defines the direction of cell motility is cofilin. Cofilin is a ubiquitous actin-binding factor required for the reorganization of actin filaments by causing depolymerization at the end of filaments and preventing their reassembly (27, 28). Talin is another actin-binding protein that links integrins to the actin cytoskeleton in focal adhesion complexes and plays a role in cell adhesion and cell motility (29, 30). To study changes in cytoskeleton organization responses to androgens, the expression of key cytoskeleton components was evaluated by Western blot analysis and immunofluorescence staining. DHT treatment of LNCaP TβRII cells led to up-regulation of β-actin and its partner cofilin, as well as the major focal adhesion effector, talin (**Fig. 2A**). Expression of α-tubulin was also significantly down-regulated (Fig. 2A). Flow cytometric analysis revealed a significant increase in actin, talin, and cofilin fluorescence density in cells after DHT treatment, compared to CSS-control cells (Fig. 2B). In addition, DHT exposure led to changes in actin cytoskeleton reorganization: prostate cancer cells exhibit more cytopodia and microvilli (Fig. 2C, red arrowheads) and share similar features with TGF-β-treated cells. In addition, a large number of cells acquire a more round morphology in response to DHT and TGF-β treatment (Fig. 2C). Exposure to DHT for 3 d enhanced the association of actin with both cofilin and talin (Fig. 2D, arrowheads). A similar association was detected after short-term exposure (10 min) to DHT (data not shown). These observations implicate an asso-ciation between the actin microfilaments with cell motility and migration in response to androgens, possibly facilitating interaction with the ECM.

Androgens and TGF-β promote prostate cancer cell migration and invasion

Exposure of LNCAP TβRII cells to either DHT or TGF-β (as single treatments) significantly enhanced cell migration. Interestingly, the DHT/TGF-β combination did not lead to a synergistic increase in prostate cancer cell migration ability after 3 d treatment (**Fig. 3A**), consistent with our observation in EMT pattern (Fig. 1C, D). We subsequently examined the effect of DHT on prostate cancer cell invasion using the Boyden chamber invasion assay. As shown in Fig. 3B, DHT enhances the invasion ability of LNCaP TβRII cells, but has no significant effect on the parental LNCaP cells, consistent with our observation that low androgen levels (1 nM DHT) failed to induce EMT in LNCaP cells, indicating that intact TGF-β signaling is required for the manifestation of the androgenic effect.

High AR content suppresses androgen-induced EMT phenotype

PC-3 cells exhibited a strong sensitivity to the EMT effect by DHT (Fig. 1A). To determine the role of AR in androgen-induced EMT, we initially evaluated AR ex-
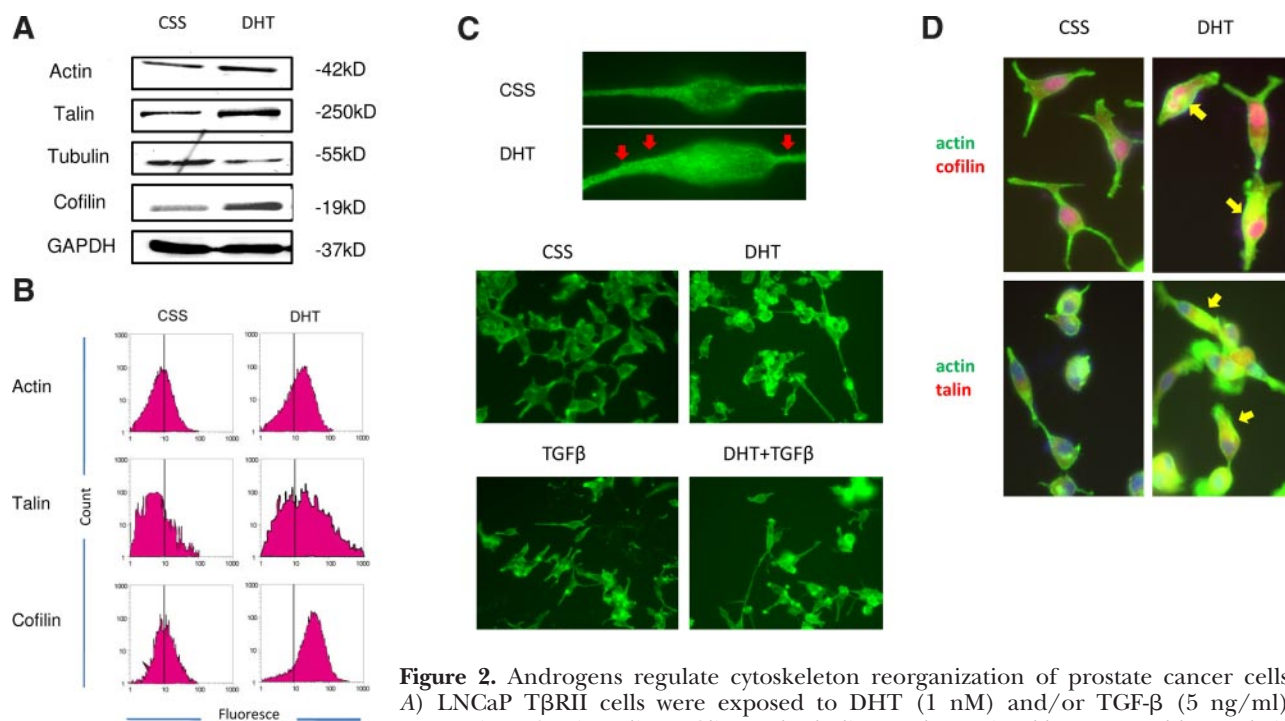

Figure 2. Androgens regulate cytoskeleton reorganization of prostate cancer cells. *A*) LNCaP TβRII cells were exposed to DHT (1 nM) and/or TGF-β (5 ng/ml). Expression of actin, talin, cofilin, and tubulin was determined by Western blot analysis. GAPDH served as internal loading control. *B*) LNCaP TβRII cells were exposed to DHT and subjected to immunofluorescence for actin, talin, and cofilin detection. Level of cytoskeleton proteins was assessed by FACS. *C*) LNCaP TβRII cells were treated with DHT and/or TGF-β, and F-actin was detected using FITC-phalloidin under fluorescent microscopy. Red arrowheads indicate microvilli formation. *D*) Actin colocalization of talin and cofilin in response to androgens (yellow arrowheads); after treatment with DHT, LNCaP TβRII cells were subjected to immunofluorescence: red indicates cofilin (top panels) and talin (bottom panels), respectively; green indicates actin; blue indicates nuclear staining; and yellow indicates colocalization.
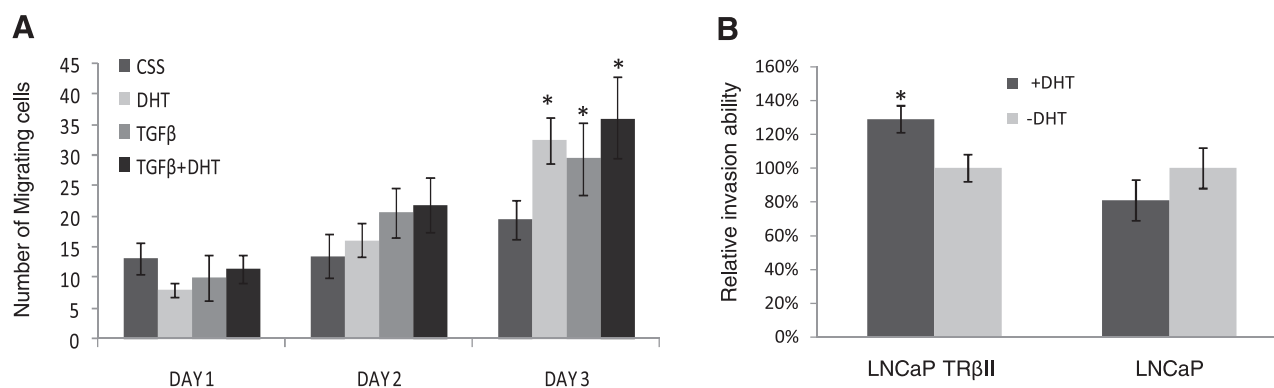
Figure 3. Effect of androgens and TGF-β on prostate cancer cell invasive behavior. *A*) LNCaP TβRII cells were treated with DHT (1 nM) and/or TGF-β (5 ng/ml) for 24, 48, and 72 h, and cell migration was determined. *B*) Effect of androgens on prostate cancer cell invasion. LNCaP and LNCaP TβRII cells were exposed to DHT for 48 h, and cell invasion was assessed as described in Materials and Methods.

pression in PC-3 cells using Western blot analysis and flow cytometric analysis. In accordance with the recent reports that AR is expressed in PC-3 cells at low levels (31, 32), we found that prolonged exposure of Western blots revealed detectable AR levels (**Fig. 4B**). This was confirmed by FACS that revealed a marked peak shift in AR immunofluorescence, compared to the isotope IgG staining control, and AR expression could be induced by the DHT treatment (Fig. 4*D*). Considering the evidence that membrane-located, nonclassical AR could be activated by androgens to elicit multiple downstream effects (33), we pursued the significance of membrane-associated AR in signaling the EMT effect. Figure 4*A* reveals that in PC-3 cells, treatment with BSA-conjugated testosterone (unable to go through cell membrane) failed to induce the EMT phenotype. However, exposure of both LNCAP TβRII and PC-3 cells to BSA-conjugated testosterone induces critical downstream signaling events, including MAPK and Src activation (Supplemental Fig. 1), implicating that nongenomic AR signaling might be involved in dictating EMT.

Considering that elevation of AR in PC-3 cells suppressed the EMT phenotype, we subsequently determined whether the EMT effect requires AR function (ligand induced). To further confirm the AR involvement in DHT-induced EMT, the EMT phenotype was profiled in the presence of the androgen receptor antagonist Casodex (10 μM) in PC-3 cells. As shown in Fig. 4*C*, DHT-induced down-regulation of E-cadherin and β-catenin (epithelial markers), and up-regulation of N-cadherin (mesenchymal marker) and vimentin (EMT marker) was abolished (Fig. 4*C*). The tissue-type specificity of androgen-induced EMT was investigated in AR-bearing MCF-7 human breast cancer cells and 786-0 renal carcinoma cells. In response to DHT, the EMT phenotype was evident in MCF-7 breast cancer cells but not in renal cancer cells (Supplemental Fig. 3).

The potential role of AR in mediating the EMT effect was investigated by introducing the wild-type (wt) AR and the mutant (mt) AR (877A mutation; with higher androgen affinity), in PC-3 cells (low endogenous AR) (**Fig. 5A**). Overexpression of either the wtAR or mtAR (LNCaP-harbored AR mutation), significantly suppressed the growth of prostate cancer PC-3 cells

(Supplemental Fig. 2). The expression pattern of E-cadherin, β-catenin, and N-cadherin was evaluated after exposure of cells to DHT (1 nM). As shown in Fig. 5*C*, DHT led to decreased expression of E-cadherin and β-catenin, and up-regulation of N-cadherin, changes characteristic of EMT, in the parental PC-3 cells, but not in AR-overexpressing cells.

Subsequent experiments examined the effect of DHT on cell migration and invasion in PC-3 AR-overexpressing cells. DHT enhanced the invasion ability in PC-3 parental cells, while there was no effect on either the wt or the mtAR-overexpressing cells (**Fig. 6A**). To trace androgen-regulated changes in the cytoskeleton reorganization in prostate cancer cells, the intracellular localization and distribution of cofilin and β-actin were determined in response to DHT. As shown on Fig. 6*C*, in response to DHT, parental PC-3 cells exhibited marked changes in the actin cytoskeleton organization and cofilin/actin colocalization, resembling the EMT characteristics, while AR-overexpressing PC-3 cells failed to exhibit any such changes.

Low AR content sensitizes prostate cancer cells to androgen-induced EMT

The AR requirement in androgen-induced EMT was examined by loss-of-expression studies. AR expression was effectively suppressed in LNCaP and CWR22 cells using the shRNA approach (Fig. 5*B*). The expression pattern of E-cadherin and β-catenin was used to evaluate the EMT effect in both LNCaP and CWR22 cells. DHT (1 nM) failed to induce EMT in the parental LNCaP or CWR22 cells (Fig. 5*D*). In cells harboring low AR content, DHT induced down-regulation of E-cadherin and β-catenin (Fig. 5*D*). Immunofluorescence analysis revealed the actin cytoskeleton reorganization and the enhancement of colocalization of actin filament and cofilin/talin in the AR-silenced cells, but not in parental control cells (Fig. 6*D*). In addition, DHT increased the invasion potential of LNCaP AR-silenced cells, while there was no significant change in the LNCaP parental cell invasion in response to DHT (Fig. 6*B*). Thus, low intracellular AR levels sensitize prostate cancer cells to androgen-induced EMT.
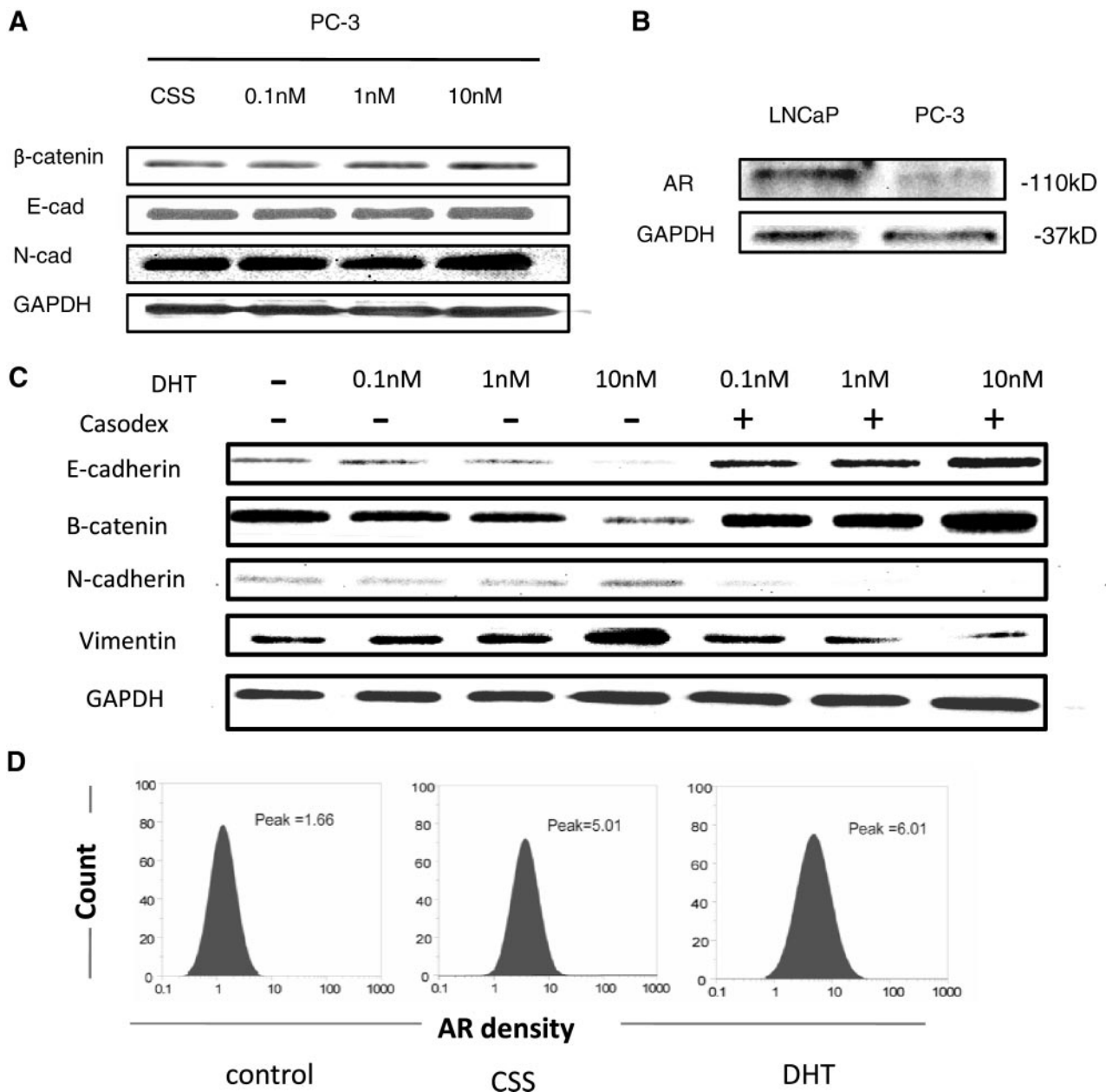
Figure 4. Detection of AR in PC-3 Cells. *A*) PC-3 cells were treated with increasing doses (0.1, 1, and 10 nM) of BSA-testosterone. Expression patterns of β-catenin, E-cadherin, and N-cadherin were evaluated by Western blotting. GAPDH was used as a loading control. *B*) AR expression in PC-3 cells after DHT treatment was detected by Western blot analysis. *C*) PC-3 cells were treated with DHT (0.1–10 nM) and Casodex (10 μM). Immunoblotting was used to assess expression of E-cadherin, β-catenin, N-cadherin, and vimentin. *D*) AR expression in PC-3 cells after DHT treatment, as determined by immunofluorescence followed by FACS analysis.

DISCUSSION

The precise role of the androgen axis and the effect of androgen-deprivation therapy in prostate cancer metastasis are still unclear. EMT is a process during which polarized epithelial cells acquire a migratory fibroblastoid phenotype and a critical event during cancer metastasis (11, 34). The hallmark of EMT is loss of expression of the cell adhesion molecule E-cadherin. E-cadherin is a cell-cell adhesion molecule that participates in calcium-dependent interactions to form epithelial adherent junctions. Prostate epithelial cells undergo EMT in response to an array of soluble factors, including TGF-β1 plus EGF, IGF-1, and β2-microglobulin (β2-m), or exposure to a bone microenvironment (35). The present findings demonstrate that androgens suppress E-cadherin expression and induce mesenchymal marker expression in prostate cancer epithelial cells. One could argue that this might facilitate escape of prostate cancer cells from the primary site and migration to distant sites, an important concept considering that activation of EMT may result in increased bone turnover, implicated in prostate cancer bone colonization in metastatic disease. Furthermore, alterations in cytoskeleton reorganization induced by an-
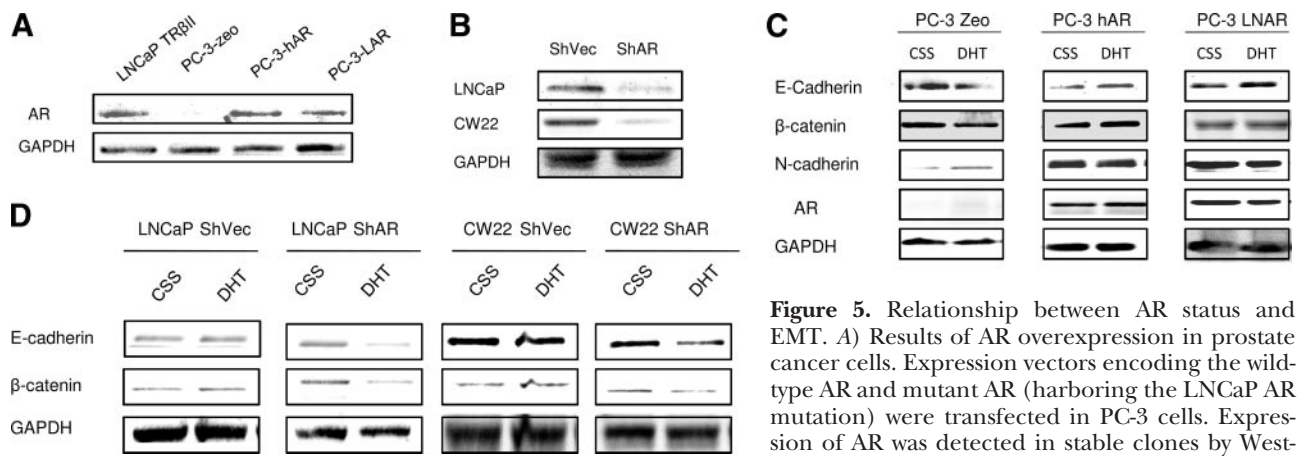
Figure 5. Relationship between AR status and EMT. *A*) Results of AR overexpression in prostate cancer cells. Expression vectors encoding the wild-type AR and mutant AR (harboring the LNCaP AR mutation) were transfected in PC-3 cells. Expression of AR was detected in stable clones by Western blot analysis. *B*) ShAR RNA was transfected into LNCaP cells and CW22 cells, and stable transfectants were generated. Reduction/loss of AR protein was examined by Western blotting. *C*) PC-3 Zeo, PC-3-hAR, and PC-3-LAR cells were treated with DHT (1 nM) and/or TGF-β (5 ng/ml). Expression of E-cadherin, β-catenin, and N-cadherin were determined by Western blot analysis. GAPDH served as internal control. *D*) Expression profile of E-cadherin and β-catenin in LNCaP-null vector control and LNCaP ShAR cells; CW22-null vector control and CW22 ShAR cells after treatment with DHT and TGF-β.

drogens may enable cell migration and metastasis of the escaped prostate tumor cells. Changes in actin microfilament network organization in androgen-treated cells could provide active movement assisting cell migration and the dynamics of interaction with adherent molecules in the ECM. Considering that the reactive prostate stroma has been assigned a critical role in the context of the tumor microenvironment in prostate cancer progression

to metastasis, AR signaling in prostate fibroblasts may function as a promoter of prostate epithelial cell proliferation (36), as well as a mediator of a functional exchange between prostate epithelial and stromal cells, thus contributing to the EMT effect during cancer metastasis (37).

The existence of crosstalk between androgen and TGF-β signaling has been established (37). Interaction of Smad4, alone or together with Smad3, with the AR in
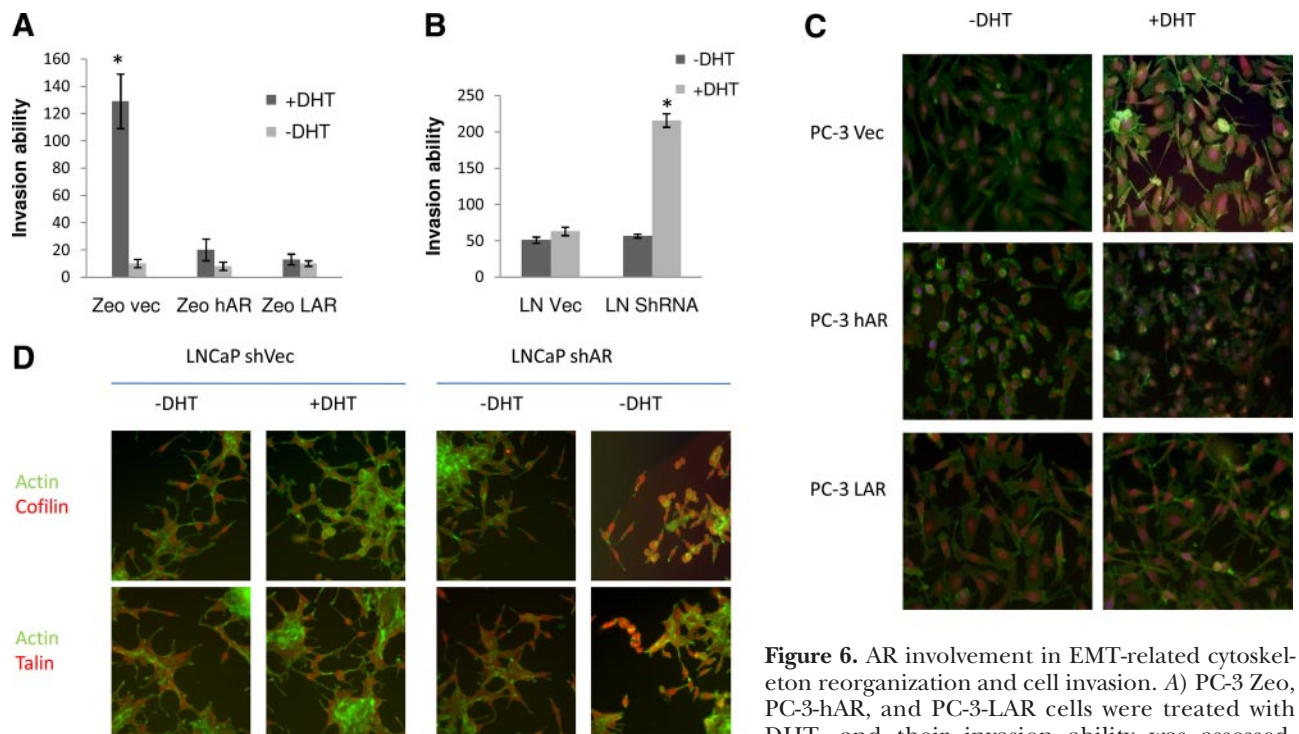

Figure 6. AR involvement in EMT-related cytoskeleton reorganization and cell invasion. *A*) PC-3 Zeo, PC-3-hAR, and PC-3-LAR cells were treated with DHT, and their invasion ability was assessed. *B*) Effect of AR loss on the invasion ability of prostate cancer cells. LNCaP-null vector control cells and LNCaP ARSh cells; CW22-null vector control cells and CW22 ShAR-silenced cells were exposed to androgens, and their invasion potential was determined. *C*) PC-3 Zeo, PC-3-hAR, and PC-3-LAR cells were treated with DHT and subjected to immunofluorescence analysis as described in Materials and Methods. Red indicates cofilin; green indicates actin microfilaments; blue indicates nuclei. *D*) LNCaP-null vector control cells and LNCaP AR sh cells; CW22-null vector control cells and CW22 ShAR cells were treated with DHT, and immunofluorescence analysis for actin (green), cofilin (red, top panels), and talin (red, bottom panels) was conducted.

the DNA-binding and ligand-binding domains, may result in the modulation of DHT-induced AR transactivation. In human prostate cancer PC-3 and LNCaP cells, Smad3 enhances AR transactivation, while co-transfection of Smad3 and Smad4 repress AR transactivation (20, 38, 39). The interaction between the androgen axis and TGF-β signaling could be the determining factor for EMT manifestation. Nuclear translocation of β-catenin has been reported in the invasive front of colorectal carcinoma (40). Moreover, β-catenin activates DNA binding protein LEF-1/TCFs to induce several signaling pathways toward mesenchymal marker expression (24). A functional exchange between AR and β-catenin results in increased nuclear colocalization and interaction of AR with β-catenin in castrate-resistant prostate tumors (41, 42). The present study suggests that activation of β-catenin by androgen signaling could serve as an alternative mechanism of androgen-induced EMT in prostate tumor epithelial cells. The involvement of several transcriptional factors (*e.g.*, zinc-finger factors Snail and Slug, 2-handed zinc-finger factors ZEB1 and SIP1, and basic helix-loop-helix factors Twist and E12/E47) in the EMT process by repressing E-cadherin expression and consequently inducing migration and metastasis has recently been documented (43, 44). Downstream activation of *Snail* by the TGF-β/Smad pathway represses E-cadherin expression in several cancer cell types (26, 45). Our results demonstrate that DHT alone or in combination with TGF-β leads to a significant increase in *Snail* expression at both the mRNA and protein level in the androgen-sensitive, TGF-β-responsive LNCaP TβRII cells, suggesting that androgens can independently induce EMT, potentially bypassing the effect elicited by TGF-β. The ability of DHT to induce *Snail* expression in prostate cancer cells by engaging a crosstalk between the androgen axis and TGF-β signaling on *Snail* transcriptional activation is currently being investigated. Further studies focus on the recruitment of β-catenin by *Snail* in EMT under conditions of androgen deprivation.

The functional outcome of EMT in prostate cancer progression to castration-resistant disease is likely to be complex, given the uncertainty surrounding the contribution of the androgen axis to prostate cancer metastasis. Indeed, the impact of androgen suppression to metastatic dissemination of prostate cancer cells is still a subject of debate, with the notion that androgen deprivation therapy may down-regulate AR in prostate tumors. One could speculate that a threshold low AR level may promote EMT, ultimately facilitating metastatic spread of prostate tumor epithelial cells. The inhibition of EMT response to androgens by AR overexpression points to an inverse relationship between AR content and EMT induction and a potential biochemical basis for the metastatic behavior of prostate cancer cells from recurrent castration-resistant tumors. Since long-term androgen deprivation may down-regulate AR expression, this threshold of "low" AR status facilitates DHT-induced EMT, thus promoting cancer metastasis. This is in accord with our observations that the AR antagonist reverses the EMT changes triggered

by androgens in prostate cancer cells, thus providing proof of principle as to the ability of elevated AR to prevent DHT-induced E-cadherin reduction and N-cadherin induction. The concept gains indirect support from the clinical evidence that intermittent androgen deprivation therapy benefits patients in prostate cancer progression (46). Emerging data from an ongoing clinical trial show intermittent androgen deprivation therapy to be a promising option for patients with locally advanced and metastatic prostate cancer, in accord with preclinical evidence, suggesting that androgen deprivation therapy (on the basis of intermittent administration) delays androgen independence (47, 48). Pulse administration can effectively target AR regulation, providing proof of principle that low AR levels induced by androgen deprivation therapy might be responsible for the more aggressive behavior of recurring prostate tumors and supporting the requirement of a threshold AR level to maintain prostate tumor growth. Gain-of-function studies have shown that activated AR (*via* mutational activation or ligand independent activation) promotes proliferation of prostate cancer cells (49, 50). In a "double-sword" twist, the present data suggest that loss of AR can actually promote prostate cancer cell metastatic ability by regulating EMT. This study provides a novel insight into the androgen-mediated EMT effect, as a biological process significantly contributing to castration-resistant prostate cancer metastasis. [FJ]

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Appendix 2

EMMPRIN[Q1] Regulates Cytoskeleton Reorganization and Cell Adhesion in Prostate Cancer

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BACKGROUND. Proteins on cell surface play important roles during cancer progression and metastasis via their ability to mediate cell-to-cell interactions and navigate the communication between cells and the microenvironment.

METHODS. In this study a targeted proteomic analysis was conducted to identify the differential expression of cell surface proteins in human benign (BPH-1) versus malignant (LNCaP and PC-3) prostate epithelial cells. We identified EMMPRIN (extracellular matrix metalloproteinase inducer) as a key candidate and shRNA functional approaches were subsequently applied to determine the role of EMMPRIN in prostate cancer cell adhesion, migration, invasion as well as cytoskeleton organization.

RESULTS. EMMPRIN was found to be highly expressed on the surface of prostate cancer cells compared to BPH-1 cells, consistent with a correlation between elevated EMMPRIN and metastasis found in other tumors. No significant changes in cell proliferation, cell cycle progression, or apoptosis were detected in EMMPRIN knockdown cells compared to the scramble controls. Furthermore, EMMPRIN silencing markedly decreased the ability of PC-3 cells to form filopodia, a critical feature of invasive behavior, while it increased expression of cell–cell adhesion and gap junction proteins.

CONCLUSIONS. Our results suggest that EMMPRIN regulates cell adhesion, invasion, and cytoskeleton reorganization in prostate cancer cells. This study identifies a new function for EMMPRIN as a contributor to prostate cancer cell–cell communication and cytoskeleton changes towards metastatic spread, and suggests its potential value as a marker of prostate cancer progression to metastasis. *Prostate 9999: 1–10, 2011.* © 2011 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; EMMPRIN; cytoskeleton; shRNA; filopodia

INTRODUCTION

Metastatic prostate cancer is a major contributor to cancer related mortality in men. Normal prostate epithelial cell homeostasis is maintained by a dynamic balance between cell proliferation and apoptosis. Normal cells undergo anoikis (a unique mode of apoptosis) upon detachment from extracellular matrix (ECM). Cancer cells however develop mechanisms to evade anoikis and acquire the ability to detach and migrate into new sites that provide a nurturing microenvironment for continued growth [1]. During the metastatic spread of primary tumor cells, proteins on cell surface are critical in mediating cell-to-cell and cell-to-environment communication.

EMMPRIN is a cell surface glycoprotein of IgG superfamily encoded by a gene localized to 19p13.3 [2,3]. EMMPRIN is an integral membrane protein, but may be released as a soluble protein by vesicle shedding [4,5]. It initiates the function through homophilic interactions between EMMPRIN molecules on neighboring cells [4,5]. EMMPRIN is expressed in numerous normal and malignant cells and mediates diverse processes such as angiogenesis, neuronal signaling, cell differentiation, wound healing, and embryo implantation [6]. Mice lacking EMMPRIN demonstrate various defects, including low embryonic survival, infertility, deficiencies in learning and memory, abnormality in odor reception, retinal dysfunction, and mixed lymphocyte reaction [6–10]. Elevated expression of EMMPRIN is found in several human cancers and correlates with the metastatic potential of tumor cells, specifically in breast and ovarian cancer epithelial cells during progression to metastasis [11–14]. In the context of the tumor microenvironment, EMMPRIN induces matrix metalloproteinase (MMP) production in stromal fibroblasts and endothelial cells as well as in tumor cells [11–13,15–17]. Elevated MMPs result in ECM degradation and subsequent detachment and metastasis of cancer cells. In addition, EMMPRIN can promote tumor cell invasion via activation of urokinase-type plasminogen activator [18], stimulate tumor angiogenesis by elevating vascular endothelial cell growth factor (VEGF) through Akt signaling [19], and causes multi-drug resistance in tumor cells via hyaluronan-mediated up-regulation and ErbB2 signaling activation [20]. EMMPRIN is implicated in metastasis via its ability to confer resistance of breast cancer cells to anoikis by inhibiting BIM [21], and its association with lipid raft or caveolae via interactions with key membrane proteins, including caveolin-1, monocarboxylate transporters, annexin II [22], and integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$ [23], all critical in the spatial distribution and activity of EMMPRIN.

Previous studies suggested that EMMPRIN expression is associated with prostate cancer progression [24,25], and loss of EMMPRIN reduces the invasion potential of human prostate cancer cells [26]. This evidence however has been correlative and little is known about the mechanistic significance of EMMPRIN in prostate cancer progression and metastasis beyond its ability to induce MMPs. In this study we profiled the EMMPRIN expression pattern in human prostate cell lines of benign and metastatic origin and characterized the function of EMMPRIN in tumor cell aggressive behavior. EMMPRIN suppression led to a significant decrease in prostate cancer cell attachment to the ECM, migration and invasion, as well as filopodia formation while it enhanced cell–cell interactions. The results provide a new insight into the ability of EMMPRIN to regulate prostate cancer cell adhesion, invasion, and cytoskeleton organization.

MATERIALS AND METHODS

Cell Lines

The HEK293 and the human prostate cancer cell lines PC-3, DU-145, and LNCaP, were obtained from the American Type Culture Collection (Manassas, VA). The non-tumorigenic benign human prostatic epithelial cells BPH-1 (derived from human prostate epithelium of benign pathology) was generously provided by Dr. Simon W. Hayward (Department of Urological Surgery, Vanderbilt University Medical Center). Cells are maintained in RPMI-1640 medium (Gibco™, Grand Island, NY), supplemented with 10% fetal calf serum (CSS), 100 U penicillin and 100-mg/ml streptomycin, at 5% CO_2 incubator at 37°C.

Western Blot Analysis

Confluent cell cultures (80%) were washed with PBS, scraped, and cell pellets were harvested. Cells were disrupted with RIPA buffer (50 mM Tris-HCl, pH7.4, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml each of aprotinin, leupeptin, pepstatin, and 1 mM Na_3VO_4). Cell lysates were centrifuged at 5,000g (15 mins), resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with the primary and secondary antibodies, immunoreactive bands were detected using a chemiluminescent approach with the ECL kit (Pierce, Rockford, IL). Membrane fractions were prepared using the protein isolation kit (Pierce). Monoclonal antibodies against EMMPRIN, ZO-2, actin, and tubulin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal antibodies against ZO-1 and AF6 were obtained from Invitrogen Zymed (San

Francisco, CA) and BD Transduction (Lexington, KY), respectively.

RT-PCR Analysis

Total RNA was extracted from cells using an RNAeasy kit (Qiagen, Valencia, CA). RNA samples (0.25 μg) were subjected to reverse transcription (RT) PCR reaction in a 20-μl volume with poly-oligoT primer. The resulting cDNA was subjected to PCR using EMMPRIN specific primers. The first set of primers started with exon 1 and ended at exon 11: EF2 (5′-ATG GCG GCT GCG CTG TTC GTG-3′) and ER11 (5′-GGA GCA GGG AGC GTC CTC GGG-3′). The second set of primers started with exon 2 and ended at exon 11: EF1 (5′-ATG AAG CAG TCG GAC GCG TCT C-3′) and ER11. GAPDH primers (5′-CAG CAA TGC ATC CTG CAC-3′ and 5′-GAG TTG CTG TTG AAG TCA CAG G-3′) were used as control in the same PCR reactions. Thirty cycles of PCR reactions were performed and each cycle included 45 sec, 94°C; 45 sec, 55°C; and 45 sec, 72°C. The PCR products were analyzed on a 1.2% agarose gel. Amplicons are purified, cloned, and sequenced by IDT (Coralville, IA).

shRNA Plasmid Construction and Transfection

Short hairpin RNA (shRNA) interference oligos, were designed using OligoEngine software (Seattle, WA) to specifically target EMMPRIN (NM_198589). Three oligos that target EMMPRIN (variant 2 mRNA) at nucleotides 98–116 (TGGCTCCAAGATACTCC-TC), 277–295 (CCATGGGCACGGCCAACAT), and 776–794 (AGGCAAGAACGTCCGCCAG), are named as 98i, 277i, and 776i, respectively. A scramble shRNA (TTCTCCGAACGTGTCACGT) was used as control. The oligos are cloned to pSUPER (neo + GFP) plasmid from OligoEngine according to the manufacturer's instruction. Plasmids were amplified in DH5α cell and confirmed by sequencing.

Subconfluent cell populations were used for transfection using the FuGENE system (Roche, Indianapolis, IN). Briefly, the plasmid and Fugene reagent were combined and incubated for 20–30 min at room temperature. After transfection (36 hr), cells were subjected to cell sorting based on GFP expression and GFP positive cells were subsequently subjected to Western blotting. Stable tansfectants were cloned under Geneticin selection (Invitrogen) (300 μg/ml), the generated clones were maintained in RPMI 1640 medium (150 μg/ml Geneticin).

Cell Viability Assay

The MTT assay (based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt,

into an insoluble formazan precipitate) was used to assess cell viability. Cells were seeded into 96-well plates (2,500 cells/well) and incubated in growth medium (18–24 hr). After incubation with the MTT solution for 4 hr, absorbance was read at A_{570} and the colorimetric reaction product was quantitated spectrophotometrically (BioTek, PowerWave XS, Winooski, VT).

Evaluation of Cell Cycle and Apoptosis

BrdU/PI (Bromodeoxy uridine and propidium iodide) method was used for the analysis of cell cycle progression and apoptosis. Cells (1×10^6/ml) were incubated with BrdU (20 mM) (60 min at 37°C), suspended in PBS, and fixed with ice-cold 95% (v/v) ethanol. Fixed cells were subsequently permeabilized using pepsin (0.04% w/v, 0.4 mg/ml in 0.1 N HCl). BrdU was probed with FITC labeled anti-BrdU (BD, San Jose, CA). Apoptosis among the different cell populations was evaluated using the terminal UTP end-labeling (TUNEL) technique. (Leica, Germany).

Cell Adhesion Assay

The ability of cells to attach to key ECM components (fibronectin and laminin) was tested using fibronectin or laminin-coated 6-well multiwell plates (BD Bioscience). Prostate cancer epithelial cells were plated (10^5/well), and incubated at 37°C for 30 min, prior to fixing with methanol, and washed with PBS. Cells were counted from three random fields/well.

Evaluation of Cell Migration and Invasion

Confluent monolayer cells were wounded by scraping. Cultures were washed twice with medium, and then incubated at 37°C for 16 hr to allow migration toward the gap. The number of migrating cells was determined under the microscope. The invasion potential of prostate cancer cells was assessed using Biocoat Matrigel invasion chambers (Becton Dickinson). Briefly, cells (5×10^4) resuspended in RPMI1640-based medium were added (250 μl) into the invasion chambers and chambers were subsequently inserted into 24-well plates. Stained cells were photographed and counted.

Confocal Microscopy

Cells were plated on fibronectin-coated glass coverslips and fixed with 4% paraformaldehyde. Cells were permeablized in 0.1% (v/v) Triton-X 100 and were subsequently stained with rhodamine-phalloidin (Jackson ImmunoResearch, West Grove, PA). After rinsing with PBS (3×), slides were mounted with Vectorshield (Vector Lab, Burlingame, CA).

Slides were examined under a laser-scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany).

Cell Aggregation Assay

Cells aggregation assay was performed as previously described [27]. Briefly, cells were suspended into single cells and dissociated cells were allowed to associate in medium (1 hr) in 5% CO_2 at 37°C, with gentle rotation of the plates. The number of cell aggregates in the parental control PC-3 and EMMPRIN shRNA transfectant cells was counted.

Statistical Analysis

Data are expressed as mean ± SD. Mann–Whitney and Student's t tests were used to comparatively analyze the differences between groups in the various experiments.

RESULTS

EMMPRIN Expression in Human Prostate Cancer Cell Lines

Targeted proteomic analysis comparing the cell surface proteomes of BPH-1 (immortalized benign prostate hyperplasia cell line) and LNCaP and PC-3 (human prostate cancer cell lines derived from metastatic lesions) revealed the differential expression of EMMPRIN. EMMPRIN was found to be highly expressed on the cell surface of prostate cancer epithelial cells but not the benign prostate cells. Western blot analysis was subsequently conducted to validate the proteomics screening data and the results are shown in Figure 1. Using total cell lysates (Fig. 1, panel A), EMMPRIN showed a broad range molecular shift corresponding to different degrees of glycosylation as previously shown in breast cancer cells [11–13]. Malignant prostate cells, PC-3 and LNCaP appeared to have more highly glycosylated EMMPRIN than BPH-1 while the total protein levels were similar in all three cell lines. Glycosylation of EMMPRIN contributes to its membrane localization. Thus, plasma membrane fractions were isolated from all prostate cell lines and subjected to Western blotting. As shown in Figure 1B, EMMPRIN levels in the plasma membrane fractions of LNCaP and PC-3 cells were significantly higher than in BPH-1 cells. These results are consistent with the cell surface proteome studies, implicating higher EMMPRIN translocation to the plasma membrane in prostate cancer cells than in benign cells. The molecular mechanism of membrane targeting and translocation is beyond the scope of this article and is currently being pursued in a parallel study.
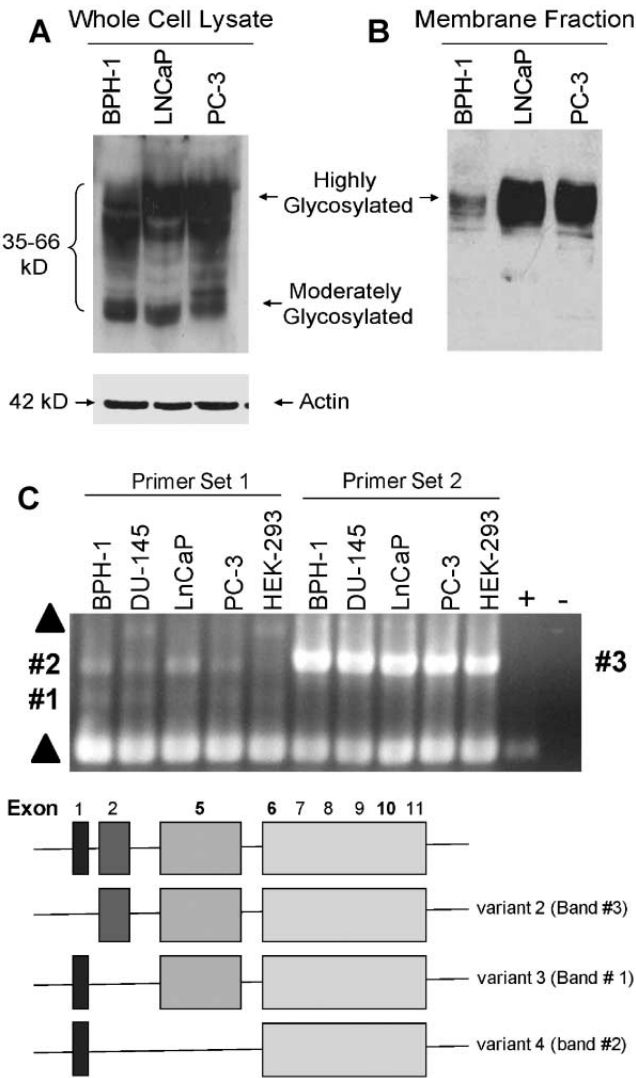

Fig. 1. EMMPRIN expression and alternative splicing in prostate cancer cells. Panel **A**: EMMPRIN expression levels in total cell lysates of BPH-1, LNCaP, and PC-3 cells. Bands of different motilities are likely due to glycosylation. Both LNCaP and PC-3 cell lines exhibited elevated levels of highly glycosylated EMMPRIN compared to BPH-1 cells. Panel **B**: LNCaP and PC-3 prostate cancer cells exhibited a significantly higher amount of membrane anchored EMMPRIN compared to BPH-1 cells. Membrane fractions (30 μg protein) were subjected to Western blotting. (**C**) EMMPRIN transcripts were analyzed by RT-PCR and electrophoresis. Splicing variants 2, 3, and 4 were confirmed by DNA sequencing as Band #3, #1, and #2, respectively.

The alternative splicing isoforms of EMMPRIN in the prostate cell lines were also determined. Four splicing isoforms of EMMPRIN have been deposited in the NCBI database and most studies focus on variant 2 that harbor two Ig domains. Two sets of primers were designed for RT-PCR: One starting at exon 1 and ending at exon 11, and the other one starting at

exon 2 and ending at exon 11. The RT-PCR products were analyzed by agarose electrophoresis and the results are shown in Figure 1 (Panel C). The RT-PCR products were cloned and subjected to DNA sequencing. The sequencing results demonstrated that there are three different splicing variants existed in human prostate cells: Variant 2 (band 3, 828 bp), variant 4 (band 1, 634 bp), and variant 3 (band 2, 793 bp). Other bands indicated by filled triangles were non-specific RT-PCR products. Variant 2 appeared to be the major transcript in human prostate cells and there were no evident differences in the splicing isoforms among the different cell lines.

EMMPRIN Silencing in PC-3 Prostate Cancer Cells

The functional significance of EMMPRIN in prostate cancer progression remains unknown. Thus we examined whether high levels of EMMPRIN in PC-3 cells, functionally contribute to the aggressive behavior of metastatic prostate cancer cells. Since PC-3 exhibits high endogenous EMMPRIN expression, we used the RNA interference approach to silence EMMPRIN in these cells. Three pairs of oligos targeting to EMMPRIN exon 5, 6, and 11 were designed and successfully cloned into pSUPER plasmid (containing GFP marker). Due to the low transfection efficiency in PC-3 cells (about 30% using FuGENE), cells with the GFP marker were sorted for 36 hr after transfection and were subjected to Western blot analysis. The results shown in Figure 2A indicate that EMMPRIN protein levels are significantly reduced by all three shRNA species. Stable clones in which EMMPRIN was silenced under G418 selection, had lower EMMPRIN levels compared to scramble controls (Fig. 2, panels A and B).The shRNA 277 clone, in which the middle region of EMMPRIN gene was targeted, had less of an effect in reducing EMMPRIN expression.

Effect of EMMPRIN Loss on Prostate Cancer Cell Proliferation and Apoptosis

To determine the role of EMMPRIN on prostate cancer cell growth, we initially examined the consequences of EMMPRIN silencing on prostate cancer cell proliferation, cell cycle, and apoptosis. Interestingly, down-regulation of EMMPRIN resulted only in a modest inhibitory effect on prostate cancer cell growth (Supplementary Fig. S1, panel A). Cell cycle analysis demonstrated no significant effect on cell cycle progression in shRNA EMMPRIN PC-3 transfectants (Fig. S1, panel B). Evaluation of apoptosis based on the TUNNEL assay revealed that loss of EMMPRIN had no significant consequences on the rate of cell death among these cell populations
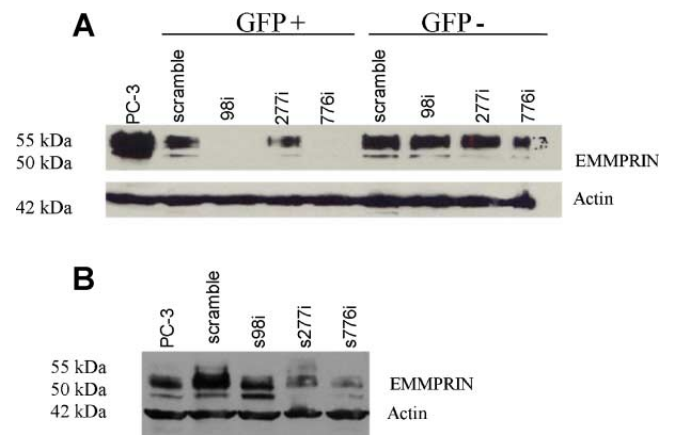
Fig. 2. Suppression of EMMPRIN expression in transient and stable shRNA prostate cancer transfected cells. PC-3 cells were transfected with three EMMPRIN shRNA plasmids or scrambled control and subjected to Western blot analysis to establish EMMPRIN protein expression levels (major band detected at 50 kDa). Panel **A**: After transient transfection a considerable reduction in EMMPRIN expression was detected in GFP-positive cells transfected with three different shRNA plasmids, while no changes in EMMPRIN levels were observed in the GFP negative control cells. Panel **B**: Stably transfected cells with GFP marker were obtained after selection with G418 (300 µg/ml). Substantial reduction in EMMPRIN levels was demonstrated in the individual stable shRNA clones.

(Fig. S1, panel C). Thus, EMMPRIN is not involved in the control of prostate cancer cell growth or apoptosis.

EMMPRIN Loss Decreases Prostate Cancer Cell Adhesion, Migration, and Invasion

Many cell surface proteins are involved in cell adhesion and EMMPRIN can be a potential partner with such adhesion molecules. To determine the functional contribution of EMMPRIN to prostate cancer cell adhesion to the ECM, we examined attachment ability of EMMPRIN silenced PC-3 transfectants to key components of the ECM, fibronectin, and laminin. As shown in Figure 3 (Panel A), there was a 40% decrease in the number of cells attached to fibronectin for the EMMPRIN knockdown cells compared to the scramble control cells. A similar magnitude of suppression of cell adhesion to laminin was observed in the EMMPRIN shRNA stable clones compared to scramble control cells (Fig. 3, panel B) or PC-3 parental cells (approximately 30–50% suppression). We subsequently examined the consequences of EMMPRIN loss on prostate cancer cell migration. EMMPRIN silencing yielded a significant reduction in cell migration ability in all three shRNA prostate cancer cell lines (Fig. 3, panel B), with the s277i clone
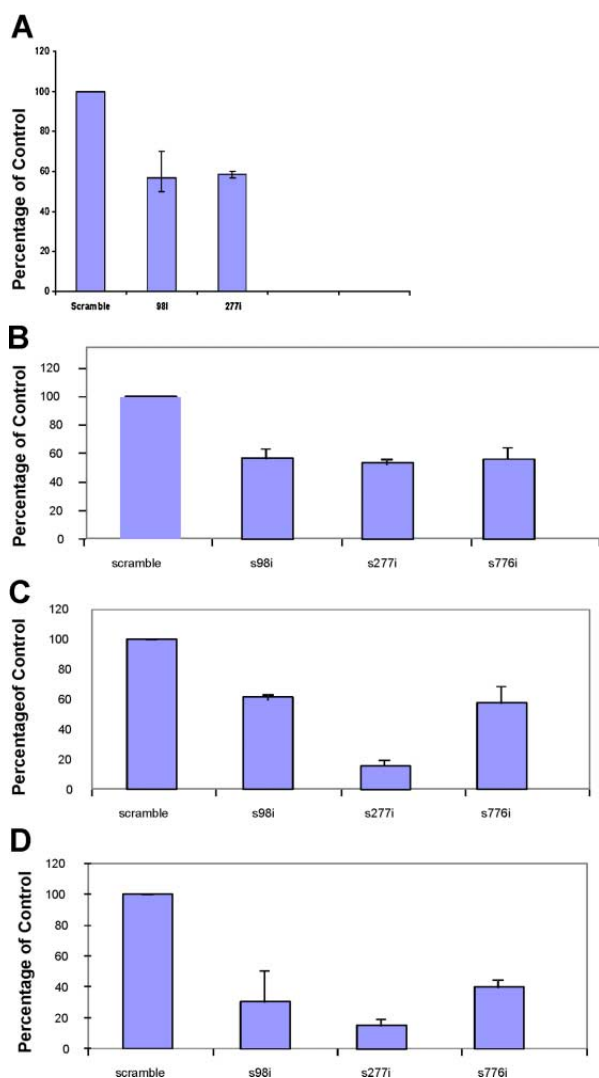
Fig. 3. Consequences of EMMPRIN silencing on prostate cancer cell adhesion to ECM, migration, and invasion. EMMPRIN shRNA transfectant PC-3 cells were seeded on fibronectin-coated (panel **A**) or laminin (panel **B**) plates for 30 min and attached cells were fixed and counted. Panel **C**: Cell migration was assessed by wounding the cell monolayer and determining the number of cells migrating to the wounded area after 24 hr. Panel **D**: The effect of EMMPRIN silencing on prostate cancer cell invasion was determined using the matrigel assay as described in "Materials and Methods" section. The average values from three independent experiments performed in triplicate are shown. Numerical values are expressed as percentage of control Sh scramble cells. Statistical significance is reached at $P < 0.01$.

exhibiting the most significant suppression. In addition, we examined the impact of EMMPRIN loss on the invasion ability of PC-3 cells. Figure 3 (Panel C) shows a significant decrease in cell invasion observed in EMMPRIN shRNA transfected cells compared to control cells. Thus, loss of EMMPRIN

significantly decreased the adhesion, migration, and invasion abilities of metastatic prostate cancer cells.

EMMPRIN Enhanced Filopodia Formation in Prostate Cancer Cells

To determine whether EMMPRIN promotes cell migration by facilitating cytoskeleton reorganization, we examined the ability of EMMPRIN shRNA PC-3 cells, to form filopodia. Cells attached to fibronectin-coated cover-slips were subjected to immunofluorescence analysis for vinculin and F-actin presence and localization. The image in Figure 4 (Panel A), indicates that EMMPRIN silencing inhibited prostate cancer cell spreading on fibronectin, while a stronger F-actin staining was detected forming a stress fiber but without typical focal adhesion complex (Fig. 4, panel A). Confocal microscopy revealed a significant suppression of filopodia formation as a consequence of EMMPRIN loss. An approximate 50% reduction in the number of filopodia is detected in EMMPRIN knockout cells compared to control cells (Fig. 4, panel B). In addition, EMMPRIN silencing also led to a decrease in the strength of the filopodia. Immunofluorescence analysis (Fig. 4, panel A) revealed considerably larger filopodia in control cells compared to limited and small filopodia observed among EMMPRIN knockdown PC-3 prostate cancer cells.

Effect of EMMPRIN Knockdown on Cell Aggregation and Tight Junction Proteins

We subsequently examined the effect of EMMPRIN on the dissociation/detachment of cancer cells. A cell aggregation assay was conducted in the PC-3 control and EMMPRIN shRNA PC-3 prostate cancer cells. As shown in Figure 5 (Panel A), there was increased cell aggregation in EMMPRIN silenced PC-3 cells. Subsequent experiments determined the effect of EMMPRIN silencing on the expression of tight junction proteins. The levels of plasma membrane proteins JAM-A and JAM-B were unchanged in the EMMPRIN knockdown clones (Fig. 5, panel B). A significant increase however in the levels of tight junction associated proteins ZO-1, ZO-2, AF6, and β-catenin was detected consequential to EMMPRIN loss. These data imply that EMMPRIN may impair cell–cell interactions by facilitating the dissociation/detachment of tumor epithelial cells from each other.

DISCUSSION

To determine the cell surface protein differences between malignant and benign prostate cells and their significance in prostate cancer metastasis, we performed mass spectrometry analysis to profile the
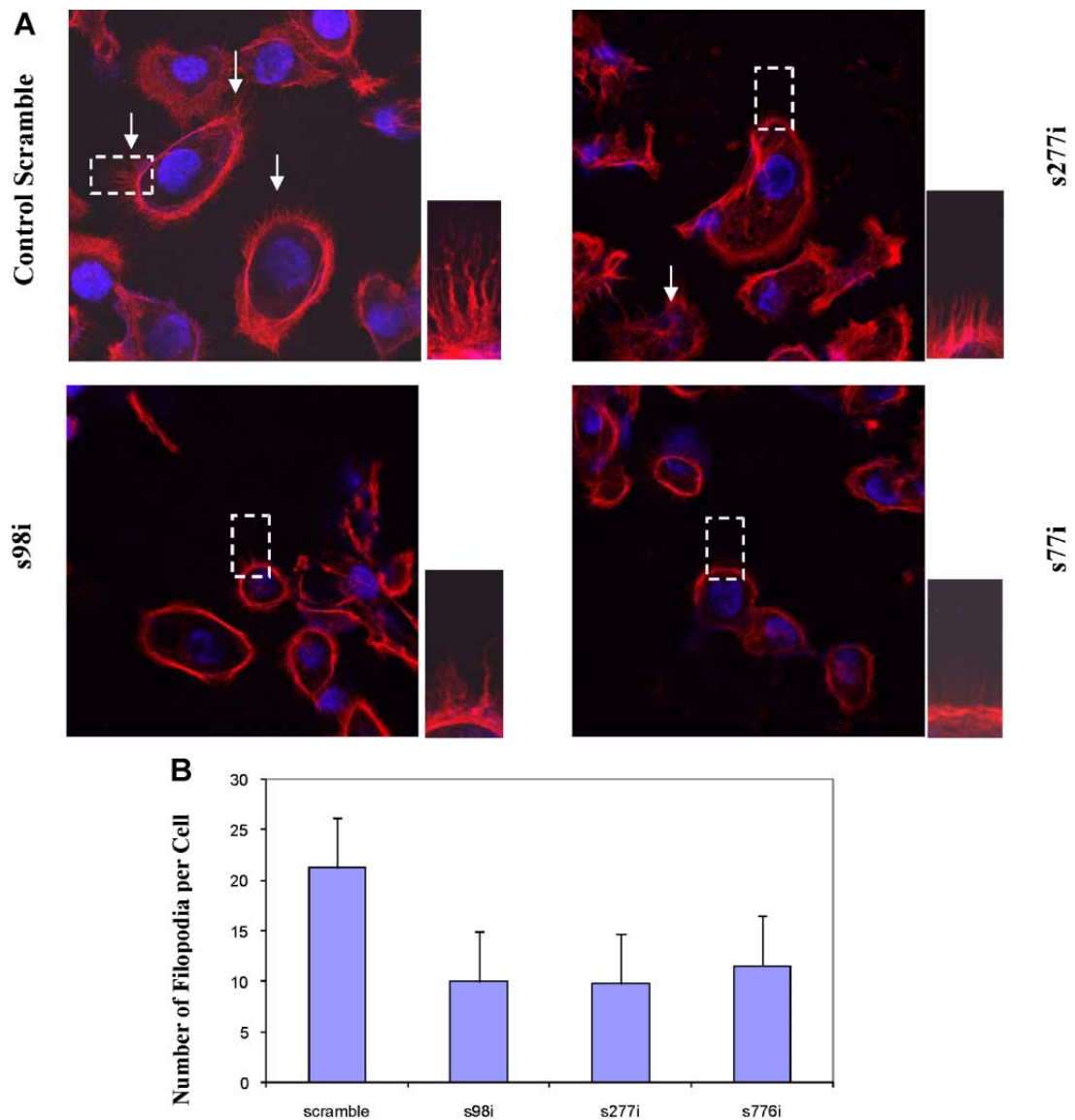
Fig. 4. EMMPRIN loss reduces filopodia formation in prostate cancer cells. Panel **A**: Cells were plated on fibronectin-coated glass coverslips and after spreading (24 hr), they were exposed to F-actin staining, and subsequently visualized under confocal microscopy; arrow heads indicate individual filopodia. The insert represents a zoom-in of region indicated in doted boxed areas. Panel **B**: The average number of filopodia in each cell was quantified in scramble control and the three shEMMPRIN clones, 98i, 277i, and 776i cells. Filopodia from at least 20 cells were counted and representative average values are shown. Approximately 20 cells/field and 10 random fields were examined for each cell line; error bars indicate average values from these measurements (mean) ± standard error of mean (SEM) gathered from three independent experiments. Statistical difference is considered significant at $P < 0.01$.

expression of cell surface proteins in human prostate cancer cells derived from metastatic lesions and benign prostate epithelial cells. One of the proteins highly expressed on the cell surface of metastatic prostate cancer cells, but not benign cells, was identified to be extracellular matrix metalloproteinase inducer (EMMPRIN, also known as basigin, CD147, OX47 or 5A11). EMMPRIN has been previously shown to be involved in cancer development via its ability to stimulate MMP production and consequently control extracellular matrix remodeling and anchor independent growth [28]. In addition, EMMPRIN has been shown to regulate angiogenesis by engaging the AKT–PIK3 pathway [19], and to up-regulate urokinase-type plasminogen activator [18]. EMMPRIN can also interact with key adhesion proteins such as integrins [23], implicating its role in cancer cell migration and invasion. The present study provides the first evidence on the functional consequences of EMMPRIN loss on prostate cancer cell growth, proliferation, apoptosis and cell adhesion (Fig. 3). We observed that down-regulation of
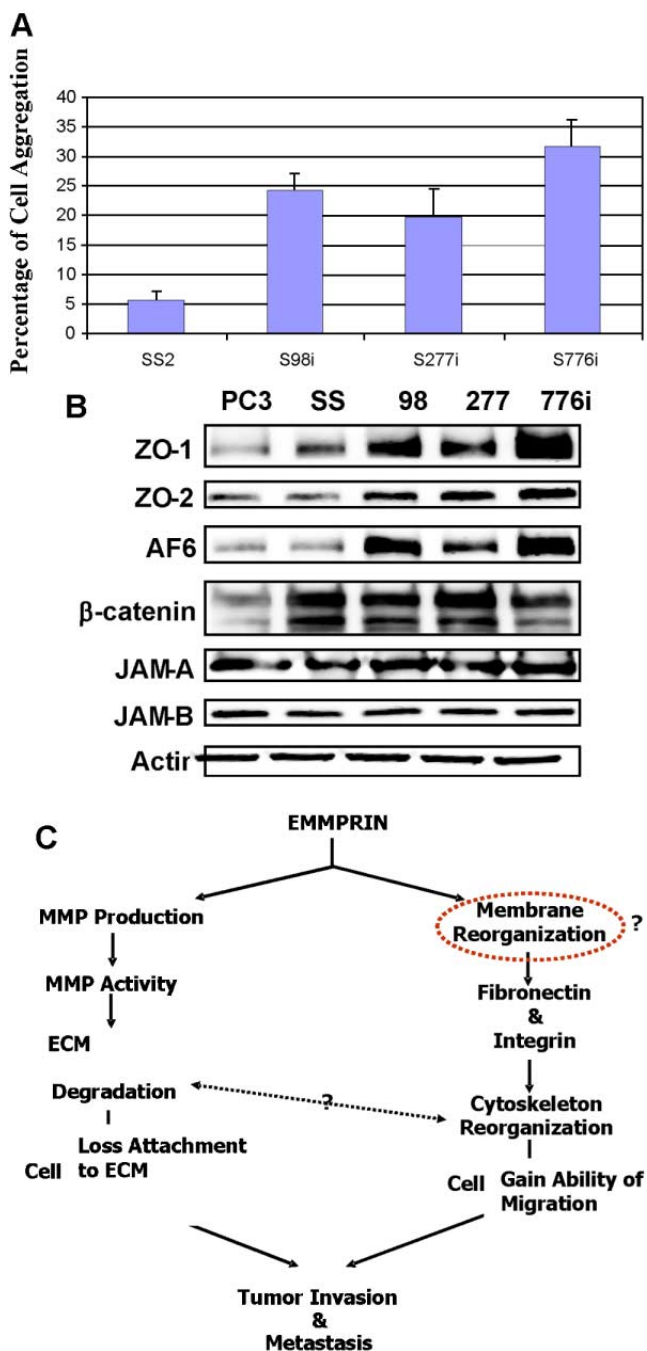
A

B

C

Fig. 5. Effect of EMMPRIN on cell aggregation and tight junction proteins. Panel **A**: Cell aggregation increased with reduced EMMPRIN expression in prostate cancer cell EMMPRIN Sh clones. Panel **B**: Western blotting indicating expression of gap junction proteins in EMMPRIN silenced PC-3 cells. Panel **C**: Potential role of EMMPRIN in prostate cancer metastasis. EMMPRIN can stimulate production of MMPs, leading to reduced cell adhesion to ECM. Alternatively, EMMPRIN may directly promote the metastatic potential of prostate cancer cells by enhancing migration and invasion through cytoskeleton reorganization and impairing cell–microenvironment interactions.

EMMPRIN led to a significant suppression of prostate cancer attachment to fibronectin, a major ECM component (Fig. 4, panel A). Thus a defect in the cytoskeleton organization can be induced by functional loss of EMMPRIN. Furthermore down regulation of EMMPRIN protein led to decreased prostate cancer cell migration. Considering the evidence that cell migration is independent of MMPs and that MMP and EMMPRIN knockout mice [29] have different phenotypes, it is reasonable to postulate that these two proteins may operate in independent pathways functionally converging downstream. EMMPRIN may be engaged in distinct signaling pathways, directly promoting the invasive behavior of prostate cancer cells towards metastasis. This notion gains support from evidence indicating lack of correlation between EMMPRIN expression and MMP activity during adult mouse mammary gland development [30]. Moreover, EMMPRIN has been shown to directly promote insoluble fibronectin assembly [21].

In this study, EMMPRIN loss significantly reduced prostate cancer cell filopodia formation on a fibronectin substratum. This defective filopodia formation implies disruption of cytoskeleton organization and actin signaling in cells lacking EMMPRIN. These observations are consistent with reports suggesting that EMMPRIN (D-basigin in Drosophila) tightly regulates cytoskeleton rearrangement in *Drosophila melanogaster* [23]. Based on the present results and the existing evidence, we propose that EMMPRIN promotes tumor cell metastasis in an MMP-dependent and -independent pathway (Fig. 5, panel C). One must also consider that EMMPRIN has been associated with prominent membrane proteins caveolin-1 and vimentin, implicating its involvement in lipid raft and control of membrane dynamics. Here we show for the first time that silencing EMMPRIN resulted in enhanced cell aggregation (Fig. 5, panel A) and increased the protein expression for several tight-junctions mediators including ZO-1, ZO-2, AF-6, and β-catenin (Fig. 5, panel B). Considering the reported relationship between tight junction proteins and cytoskeletal changes associated with cell aggregation [27,31] our findings provide new insights into the ability of elevated EMMPRIN to navigate tight junctions and cell–cell adhesion within the tumor microenvironment. The mechanistic scenario discussed above can lead to enhanced prostate cancer invasiveness by EMMPRIN overexpression. Significantly enough, our group recently demonstrated that talin1, an actin-binding protein that links integrins to actin cytoskeleton in focal adhesion complexes, correlated with prostate cancer progression to metastasis [32]. Mechanistically, talin1 binding to β integrin recruits the focal adhesion partners ILK, FAK, and SRC, and

activates downstream signals, PI3K/Akt, and Erk; activation of this signaling promotes cell survival, migration and invasion, and resistance to anoikis. EMMPRIN may serve as an upstream partner for talin, facilitating its role in anoikis resistance and actin cytoskeleton remodeling, and consequently promoting metastatic spread.

Mammalian cells ubiquitously adopt a variant splicing strategy to cope with multiple functions and their requirement by diverse physiological processes. At least two different variants of EMMPRIN have been reported. Variant 2 is a ubiquitous expression protein as previously reported and a larger variant 1 is expressed in retinal epithelial cells in a tissue specific fashion. In this study, we identified three distinct EMMPRIN splicing variants: Variants 2, 3, and 4 (Fig. 1, panel C). The latter two variants are distinct from the commonly found variant 2. Significantly enough these two variants lack exon 2 where glycosylation occurs [3]. Moreover, variant 4 lacks exon 5, where another glycosylation site is also located. The dynamics of the ratio of different isoforms and the mechanisms via which the different splicing variants are engaged to navigate EMMPRIN expression and activity to meet the physiological demands of both ECM remodeling and cancer cell motility are currently being pursued.

The present results are of translational significance as functional exploitation of EMMPRIN in prostate cancer metastasis may lead to new approaches for impairing the metastatic process by (a) reversing the ability of tumor cells to resist anoikis (thus enhancing their sensitivity to anoikis-inducing agents); and (b) interfering with the tumor cell migration and adhesion to secondary sites. Ongoing studies focus on immunoprofiling EMMPRIN expression in human prostate specimens from patients with primary and metastatic tumors to determine the significance of EMMPRIN as a marker of progression to advanced castration-resistant disease.

In summary, our findings demonstrate that EMM-PRIN loss has a major impact on cell membrane re-organization and spatial disruptions that significantly affect prostate tumor cell adhesion, migration, and invasion. The present work provides new insights into the function of EMMPRIN as a contributor to prostate cancer cell metastatic behavior and its potential value as a therapeutic target during tumor progression.

ACKNOWLEDGMENTS

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